


FORM PTO-1190 (REV 11-94)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER  <b>P04334US0</b>	
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>					
INTERNATIONAL APPLICATION NO. <b>PCT/CA98/00648</b>		INTERNATIONAL FILING DATE <b>2 JULY 1998 (02.07.98)</b>		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/446711</b>	
PRIORITY DATE CLAIMED <b>3 JULY 1997 (03/07.97)</b>					
TITLE OF INVENTION <b>METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS</b>					
APPLICANT(S) FOR DO/EO/US <b>ALLARD, ET AL</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.					
2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.					
3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).					
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.					
5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))					
a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).					
b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.					
c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).					
6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).					
7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))					
a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).					
b. <input type="checkbox"/> have been transmitted by the International Bureau.					
c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.					
d. <input type="checkbox"/> have not been made and will not be made.					
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).					
9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).					
10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).					
Items 11. to 16. below concern document(s) or information included:					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.					
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.					
13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment.					
<input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.					
14. <input type="checkbox"/> A substitute specification.					
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16. <input checked="" type="checkbox"/> Other items or information:      - <b>Certificate of Express Mailing (in duplicate)</b>					

U.S. APPLICATION NO. (if known, app. 37 CFR 1.51)		INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/446711		PCT/CA98/00648	P04334US0
17. <del>XX</del> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. .... \$970.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$840.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$760.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00  ENTER APPROPRIATE BASIC FEE AMOUNT =			CALCULATIONS PTO USE ONLY
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			\$ 840.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	- 20 =		X \$18.00
Independent claims	- 3 =		X \$78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00
TOTAL OF ABOVE CALCULATIONS =			\$
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).			\$
SUBTOTAL =			\$
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			\$
TOTAL NATIONAL FEE =			\$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property			\$
TOTAL FEES ENCLOSED =			\$840.00
			Amount to be: refunded
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a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>840.00</u> to cover the above fees is enclosed.			
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c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>26-0084</u> . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
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Serial No. or Patent No: \_\_\_\_\_

Filed or Issued: \_\_\_\_\_

For: METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
 37 CFR 1.9(d) AND 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

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ADDRESS OF ORGANIZATION Pavillon Athanas David, D-3602

1430 St-Denis, Montreal, Quebec H2X 3J8 CA

TYPE OF ORGANIZATION

☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))

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UNITED STATES OF AMERICA

(NAME OF STATE \_\_\_\_\_)

(CITATION OF STATUTE \_\_\_\_\_)

☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE

(26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

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OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

(NAME OF STATE \_\_\_\_\_)

(CITATION OF STATUTE \_\_\_\_\_)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 36, United States Code with regard to the invention entitled METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS by inventor(s) France Allard, Fathey Sarhan and Mario Houde as described in:

☐ the specification filed herewith.

☒ application serial no. \_\_\_\_\_, filed December 22, 1999

☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention overruling to their status as small entities. (37 CFR 1.27).

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of payment, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Y PARENT, PIERRE

TITLE IN ORGANIZATION SECRETARY - GENERAL

ADDRESS OF PERSON SIGNING 1430 ST-DENIS, MONTREAL, QUEBEC H2X 3J8

SIGNATURE [Signature]

DATE February 18 - 2000

**Title of the invention**METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS**Field of the invention**

5           The invention relates to a method to increase the cold or freezing tolerance of plants by cold acclimating the plants and/or by treating the same with betaines.

          This invention also relates to the inhibition of the growth or the reduction of the growth rate of plants by treating them with betaines.

10           This invention further relates to the improvement of the germination rate of plant seeds at cold temperatures by treating the same with betaines.

**Background of the Invention**

          Betaine is a non-toxic osmolyte that is thought to play a role in the protection against environmental stresses in particular salinity and drought stress (1, 2). This compound is mostly synthesized in the chloroplast by the enzymes choline monooxygenase and betaine aldehyde dehydrogenase (1). It may accumulate in  
15           different cellular compartments to adjust the osmotic balance (3) and increase the stability of protein tertiary structure thus protecting proteins from denaturation (4). *In vitro* studies have shown that betaine can protect membranes of *Beta vulgaris* roots against heat denaturation (5). Several higher plant enzymes were also shown to be  
20           protected by betaine from denaturation caused by heat (6) NaCl or KCl (7). Betaine can also stabilize the photosynthetic activity of isolated chloroplasts over time (8) and protect photosystem II against the inhibitory effect of NaCl (9). Interestingly, it was shown that an exogenous application of 25 mM betaine on barley leaves improves recovery after an osmotic stress imposed by polyethylene glycol (-10 bar) (10).

25           Because betaines have been shown to provide some protection to plants from stressful environmental conditions they have been used to treat soils, plants and seeds.

          WO 95/35022 discloses a method for treating seeds with betaine to enhance seedling growth and protect seeds against adverse environmental  
30           conditions. The seeds may be soaked and dried or coated with betaine. The adverse conditions enumerated are water stress, excess NaCl, extreme temperature or pH and heavy metal toxicity. What is not taught are the temperature extremes and the benefits with respect to the rate of germination at low temperatures.

35           WO 96/07320 discloses the application of betaine to improve the yield of grapevines the temperature extremes are between 3°C to 30°C.

In WO 96/41530 and WO 96/41532, different compositions of betaine are disclosed for use in protecting wheat, potato and grapevines against adverse conditions including temperatures between 3°C and 30°C.

These references are silent with regards to freezing temperatures and cold acclimation, and they do not teach the advantage of combining cold acclimation with betaine accumulation in order to improve cold or freeze tolerance.

In France Allard's thesis, it is taught that in the wheat cultivar Fredrick, low temperature exposure for three days at 6°C/2°C (day/night) following the addition of 1000 mM betaine resulted in an initial improvement in survival of the plants as tested by measuring the survival rate at -10°C. The survival rate was shown to be 83%, whereas, when treated with only 1000 mM betaine, the survival rate was 51% when compared to controls. The high amount of betaine used (1000 mM) was shown to have a toxic effect to the plant since it was found that it produces chlorosis of the leaves. In these experiments, the plants did not show complete recovery and died one week after the freezing test. This reference does not teach us the conditions for increasing freezing tolerance, since this experiment did not show an actual increase in freezing tolerance as known in the art. An increase in freezing tolerance is determined by the actual increase of the  $LT_{50}$  (temperature at which 50% of the plants are killed). In the case of Allard's, there is no mention about an increase in  $LT_{50}$ . It is also worthwhile to mention that the aim of Allard's experiment was not to acclimate the plants but to use low temperature exposure for three days purposely to reduce the growth rate and keep the endogenous level of betaine concentration at a high level. Thus the initial higher survival observed is attributed to the higher betaine content. Cold acclimation is actually defined as a low temperature treatment that results in an increase in freezing tolerance of plants. In Allard's reference, it was clearly stated that three days of low temperature exposure did not result in any increase of freezing tolerance. This reference clearly does not teach us the combined effect of cold acclimation and betaine treatments on freezing tolerance. It further does not teach the effect on cultivars that genotypically exhibit less (lower) freezing tolerance. The decrease in plant growth rate is also described and it is shown to be directly proportional to the amount of betaine administered. When the maximal amount of 1000mM was applied to the cultivar Fredrick, a 29% decreased growth rate occurred when compared to control plants. Finally, in this document there were also teachings relating to the protein WCOR 410. It was taught that the protein WCOR 410 accumulates when plants are subjected to cold-acclimation or by increasing amounts of exogenous betaine. This reference however does not appear to teach the combination of cold-acclimation and betaine on the expression of the *Wcor 410* gene that leads to the improvement of freezing tolerance.

In the patent publication CA 2,104,142, the present inventors disclose the isolation and sequence of three genes responsive to cold temperature. One of these genes is *Wcor410*. However what is not taught is that the protein WCOR410 is induced by betaine in a manner proportional to the amount of betaine applied and that this protein is involved in promoting freezing tolerance in some plants. It does not teach the benefits of combining cold acclimation and betaine administration.

Kishitani et al. (11) and Koster et al. (26) teach the accumulation of betaine in plants during cold acclimation. These references do not teach the combination of an exogenously administered betaine and of cold acclimation, and this for the purpose of improving cold or freeze-tolerance.

Patent publications US 4,360,465 and SU-A-369,887 disclose that betaines are capable of suppressing vertical plant growth at concentrations in the range of 1  $\mu$ M to 1 mM. Analysis of these documents indicate that betaine was used in the hydrochloride form at a very low concentration to achieve reduced growth. In this case, the growth inhibition was due to the acidity of HCl and not to betaine. Betaine in concentrations of 1  $\mu$ M to 1 mM has no effect on plant growth. If the authors of the reference SU-A-369,887 had buffered their betaine or used a betaine free base, they would not have observed the claimed effect. In support of this argument, the present inventors observed that all the compounds used in this publication are either the HCl or sulfonium forms (the latter producing sulfuric acid). It is obvious that the observed effect on growth is due to acidic conditions rather than to the betaine compounds themselves. In conclusion, any form of acid solutions would get the same effect and there is no way in which this information could help in predicting that higher betaine concentrations would result in reducing the growth rate of plants.

USP 4,032,325 teaches the herbicide effect of betaines. There is no mention of using betaines for improving cold tolerance.

### Statement of the invention

There is now provided a method of increasing cold or freezing tolerance in a plant, which comprises the steps of:

- 5           -       acclimating said plant to a temperature higher than about 0°C but not lower than the coldest temperature that said plant is capable to withstand, for a time sufficient to induce an optimal cold or freezing tolerance, in said plant, and
- 10          -       administering betaine or a derivative thereof such as glycine betaine to said plant, in a dosage regimen sufficient to induce the same or different optimal cold or freezing tolerance in said plant;

whereby combined steps of cold-acclimating and administering betaine or derivative thereof increase cold or freezing tolerance of said plant over and above the optimal cold or freezing tolerance induced by each step alone.

15           Preferably, the dosage regimen does not provide an unacceptable toxicity, more preferably, it is non-toxic to said plant.

Any plant could benefit from such a method, preferably, rosaceae, gramineae and grasses, more preferably, roses, strawberry, golf turf, barley or wheat.

20           In the two latter plants, the time for cold-acclimating is about four weeks, and the dosage regimen is growing the plants in the presence of a solution of glycine betaine having a concentration lower than about 500 mM, preferably about 250 mM.

25           In the spring wheat variety Glenlea, in which the optimal freezing tolerance, expressed as the temperature where fifty percent of a plant population die (LT<sub>50</sub>) is about -8 °C for each step alone, the combined treatment resulted in an increase of freezing tolerance by about 6°C to reach a LT<sub>50</sub> of about -14°C and further resulted in improving photosynthetic capacity and overall physiology of the plants at cold or freezing temperatures.

The optimal freezing tolerance induced by said each step alone and/or in combination is due at least in part to an increased expression of the gene *Wcor410*.

30           This invention also relates to the reduction of the growth rate of a plant by at least 30%, which comprises the step of treating the plant with an effective dosage regimen of betaine or derivative thereof which is not lethal, preferably non-toxic to the plant.

When growing the spring wheat variety Glenlea, in the presence of 500 mM of glycine betaine for four days, the growth rate thereof was reduced by about 75%.

35           Another aspect of the present invention is a method of inhibiting the growth of a plant, which comprises the step of treating said plant with a high dose regimen of betaine or derivative thereof, which may even result in a herbicidal effect.

Another aspect of the present invention is a method of improving the germination rate of plant seeds at a temperature which is higher than about 0°C but

not lower than the coldest temperature that said plant seeds can withstand, which comprises the steps of administering to said seeds an effective dosage regimen of betaine or derivative thereof, and allowing said seeds to germinate at said temperature.

## 5 Description of the invention

This invention is described hereinbelow by way of specific embodiments and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

### Brief description of figures

10 **Figures 1a) and b).** Effect of betaine on FT in the cultivar Glenlea.

Plant survival was determined by the regrowth test as described by Perras and Sarhan (25).

**Figure 1a):** The survival was evaluated after freezing at -8°C.

15 **Figure 1b):** The  $LT_{50}$  was evaluated after freezing different samples to various temperatures

**NA**, 12 day-old control non-acclimated plants; **100**, **250**, and **500**, plants treated for 4 days with 100, 250, and 500 mM betaine at 25°C respectively; **CA**, plants cold-acclimated at 6/2°C for 30 days; **CA100** and **CA250**; plants cold acclimated at 6/2°C for 30 days in the presence of 100 and 250 mM betaine respectively. Standard deviation did not exceed  $\pm 10\%$ .

20 **Figures 2a) and b).** Effect of betaine on  $LT_{50}$  in the cultivar Glenlea

**Figure 2a):** The freezing test was performed at -8°C.

**Figure 2b):** The freezing test was performed at -13°C.

25 **NA**, 12 day-old control non-acclimated plants; **250** plants treated for 4 days with 250 mM betaine. **CA**, plants cold-acclimated at 6/2°C for 30 days; **CA250**; plants cold acclimated at 6/2°C for 30 days in the presence of 250 mM betaine.

**Figure 3.** Accumulation of the WCOR410 protein in response to different betaine concentrations in the spring wheat cultivar Glenlea.

30 Total proteins (5  $\mu$ g) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with the anti-WCOR410 antibody. **NA**, 12 day old control non-acclimated plants; **100**, **250** and **500**, plants treated for 4 days with 100, 250, and 500 mM betaine respectively; **CA**, Cold acclimated plants.

35 **Figure 4a).** Golf turf was treated three times at a weekly interval in fall with 1 L/m<sup>2</sup> of 200 mM betaine free base in water. The control area was sprayed with water only. The figure shows the turf regrowth in following early spring for the non-treated control and the betaine-treated area. The upper view shows the treated area. Notice the rapidity



of regrowth and greening in the betaine-treated area. This result reflects a greater and healthier winter survival of betaine-treated turf.

**Figure 4b).** Close up caption showing the betaine-treated area (upper view) and the non-treated control (bottom).

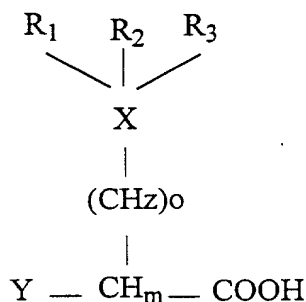
- 5 **Figures 5a) and b).** Another example of the effect of betaine on winter survival and regrowth in early spring. The betaine treatment was the same as in Figure 4 except that the application was started earlier in previous fall. Both areas were aerated at the same time. Notice the higher turf density of the betaine-treated area.

**Figure 5a).** Betaine-treated.

- 10 **Figure 5b).** Control sprayed with water.

Betaine usually refers to amino acids where the nitrogen is fully or partly methylated. Betaines are natural products present in plants and animals with a probable function as an osmolyte regulator that protect the cell from osmotic stress.

Betaines and derivatives thereof have the general formula:



15 wherein X is N or S

z is 1 or 2

o is 0 or 1

m is 1 or 2

R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> are independently void or hydrogen or methyl

20 Y is void or Q-CH<sub>w</sub>-

wherein w is 1 or 2,

Q is a molecule of 2 to 9 carbon atoms comprising or not a ketone or a hydroxyl group, which may comprise 1 or 2 nitrogen or sulfur atoms and which may form a heterocycle alone or with X.

25

Many different betaines are known and some examples are presented in Table

1.

TABLE I

	NAMES	OTHER NAMES
	Glycinebetaine	Oxyneurin, betaine
	$\beta$ -alaninebetaine	Homobetaine
5	2-trimethylamino-6-ketoheptanoate	
	Prolinebetaine	Stachydrine
	L-Proline	
	<i>Trans</i> -4-hydroxy-N-methyl-L-proline	
	<i>Cis</i> -3-hydroxy-N-methyl-L-proline	
10	(-)-4-hydroxyproline betaine	Betonicine
	(+)-4-hydroxyprolinebetaine	Turicine
	3-hydroxyprolinebetaine	3-oxystachydrine
	Histidinebetaine	Herzynine, Ercinine
	Tryptophanbetaine	Hypaphorine
15	2-mercaptohistidine-betaine	Ergothioneine
	Pipecolabetaine	Homostachydrine
	Nicotinic acid betaine	Trigonelline

20 Using two wheat cultivars that differ in their levels of freezing tolerance (FT), the role of endogenous betaine was investigated during cold acclimation. In addition, studies on the effect of an exogenous application of betaine on FT alone and in combination with cold acclimation, on the expression of low temperature-responsive genes and on photosynthetic activity have been conducted.

25 To determine if betaine accumulation is associated with increased FT, the betaine contents were determined in two wheat varieties differing in their FT (cv Glenlea,  $LT_{50}$  (lethal temperature for 50% of the plants) of  $-8^{\circ}\text{C}$  and cv Fredrick ( $LT_{50}$  of  $-17^{\circ}\text{C}$ ). In both cultivars, betaine content decreases during growth at the non-acclimated temperature of  $24/20^{\circ}\text{C}$  while it increases during growth at the cold-acclimating conditions of  $6/2^{\circ}\text{C}$ . The basal betaine level is 30% higher in the more tolerant cultivar Fredrick before cold acclimation ( $8.5 \mu\text{mol/g FW}$  (fresh weight) in  
30 Fredrick compared to  $6.5 \mu\text{mol/g FW}$  in Glenlea). At the end of the acclimation period (where maximal  $LT_{50}$  has been reached) cv Fredrick has accumulated  $21.3 \mu\text{mol/g FW}$  of betaine compared to  $15.3 \mu\text{mol/g FW}$  for cv Glenlea. On a dry weight basis, cv Fredrick has accumulated  $106.5 \mu\text{mol/g DW}$  (dry weight) compared to Glenlea which has accumulated  $82.7 \mu\text{mol/g DW}$ . This result suggests that the increase in betaine  
35 content is associated with the development of FT of the two cultivars. A similar increase in betaine was correlated with the FT of different barley cultivars (11). If we calculate the contribution of betaine to the total osmolality of the cell, we find that betaine accounts for only 3.6% and 4.5% of the osmolality after 30 days of cold

acclimation for Glenlea and Fredrick respectively. This result demonstrates that betaine contribution to the total osmolality is very low. However, as suggested by Wyn Jones *et al* (12), such a low concentration would require compartmentation in order to play a significant role as osmoprotectant. Studies performed by Matho *et al* (13) have shown that betaine is excluded from vacuoles of spinach leaf cells and is mostly found in the cytoplasm and chloroplasts. It was estimated that betaine concentration can reach 300 mM in spinach (14) and *Sueda* (8) chloroplasts when plants are submitted to salt stress. This concentration is approximately 20 fold greater than the average betaine leaf concentration. Betaine compartmentation was not determined in wheat but if we consider a similar concentration factor in the chloroplasts during cold acclimation, the actual concentration of betaine could be very significant. Since we have estimated that betaine accounts for 4.5% of the osmolality in cold-acclimated Fredrick, a twenty fold higher concentration of betaine in the chloroplast would mean that betaine contributes for approximately 90% of the chloroplasts' osmolality (or 612 mOsm). Such a

concentration could have a great impact on chloroplast function since *in vitro* studies have shown that betaine can increase the thermal stability of photosystem II (PSII; (5)) and can protect against the inhibitory effect of NaCl (9). Krall *et al* (16) have shown that betaine can stabilize the active tetrameric form of phosphoenolpyruvate carboxylase which normally forms inactive dimers when exposed to low temperature. Betaine accumulation in the chloroplasts may be an important factor that could play a significant role in maintaining chloroplast function at low temperature. It is worth noting that hardy cereals such as rye, wheat, and barley have higher basal levels of betaine compared to sensitive species such as rice, millet, and sorghum (11).

To determine whether exogenous betaine could play a role in improving FT and photosynthesis at low temperature, we first evaluated the plant's capacity to accumulate betaine. In the first experiment, we incubated plants in 500 mM betaine and determined the osmolality of the leaves at different periods. The osmolality was found to increase rapidly during the first two days and levelled off thereafter (result not shown). We repeated the experiment using different concentrations of betaine and quantified the amount of betaine accumulated in the leaves after a four day period. The method described in (17) was used to extract betaine from 1 g of leaf tissue. Quantitation was performed according to Lerma *et al* (18). For accurate evaluation, an internal standard was added before the extraction procedure. The betaine content was expressed in mOsm/kg H<sub>2</sub>O considering the tissue water content for each sample (an average of 82% water content was obtained). Osmolality was measured from leaf tissue after grinding with a mortar and pestle. The liquid obtained was centrifuged at 12,000 g for 10 min at 4°C. The osmolality was evaluated in the supernatant using a Wide Range Osmometer. We found that betaine accumulated efficiently at all concentrations used. The accumulated betaine (expressed in mOsm/Kg H<sub>2</sub>O) was equivalent to 62% of the external betaine when exposed to betaine concentrations ranging from 118 to 590 mM (100 to 500 mM). Betaine could accumulate even more at higher concentrations, however, signs of chlorosis at the leaf tips became evident at 500 mM. Chlorosis became even more extensive when higher betaine concentrations were used.

Betaine accumulation reduced the growth rate in a manner proportional to the amount of exogenous betaine applied. At the highest concentration used, the growth was reduced by 75% over the 4 day incubation period compared to control plants. The reduction in growth and more importantly, the increase in cellular betaine content was found to be associated with a substantial increase in survival rate after freezing compared to control non-acclimated plants (Fig. 1A). Interestingly, both cultivars are protected by betaine with only a slight advantage in the more tolerant cultivar at all concentrations used (not shown). Plant survival is increased even when a relatively low concentration of betaine is used. At 100mM, survival improved by 5-6 fold compared

to the untreated plants (Fig. 1A). Treating with 250 mM betaine alone was sufficient to increase the FT of the spring cultivar Glenlea from -3°C to -8°C. This value corresponds to the maximal FT achieved by this cultivar after 4 weeks of cold acclimation (Fig. 1B). Increasing the concentration of betaine to a higher concentration resulted in a slightly higher survival rate (corresponding to 55% survival at 500 mM betaine; Fig. 1A) but due to the toxicity, of higher betaine concentrations, the latter were eliminated in other experiments. We have examined whether treatment with betaine during cold acclimation could improve FT in the less tolerant cultivar Glenlea submitted to cold-acclimating conditions. Figs. 1A and 1B show that the survival of plants treated with betaine during cold acclimation were dramatically improved over plants that are cold acclimated in the absence of betaine. Fig. 2 shows the results of a typical experiment for plants treated with betaine at 25°C or during cold acclimation. Betaine treatment at 25°C for 4 days allowed the plants to reach an LT<sub>50</sub> of -8°C (the maximal LT<sub>50</sub> normally achieved by this cultivar) while those treated with betaine during cold acclimation were barely affected by a temperature of -13°C (the average LT<sub>50</sub> was estimated as -14°C in Fig. 1B). These results demonstrate that the improvement in FT observed in control plants exposed to betaine is additive in cold-acclimated plants. This finding is of crucial importance since it is the first time that the normal genotypic potential to tolerate freezing has been improved so dramatically. We have also evaluated the capacity of betaine to improve FT in barley which was also shown to accumulate betaine upon cold acclimation and found that the combined treatment of low temperature and betaine was as efficient in this species as in wheat to improve FT.

It is therefore expected that the improvement in cold or freezing tolerance will be observed in almost all plants, particularly gramineae or grasses, more particularly cereals such as rice, corn, rye, wheat, barley and oat. The conditions at which such improvement will occur are set as follows. The plants are acclimated at the coldest temperature that they can withstand. Betaine is administered before, during and at the end of the cold acclimation. The dosage regimen of betaine is determined on test plants in order to evaluate the doses at which betaine is toxic, in such a way that unacceptable toxic doses will be further avoided. We expect that even tropical plants which are very cold-sensitive will benefit from a combined treatment.

Furthermore, since the growth rate of the plants were reduced down to 25% the control plants, it is readily apparent that, if betaine is applied at the end of cold acclimation (in field, that would mean during fall wherein the maximal growth is attained), this effect would not be deleterious to the plants. Moreover, this property may be advantageously used at the beginning or during the growing period, to slow down the growth of many plants. Thus, it could be used as a growth-retarding substance for several agronomical applications. A specific useful application would be found for golf courses to reduce the maintenance costs (it would reduce the number

of times one has to mow the grass during a season). At higher toxic or lethal doses, betaine could event be used as a herbicide to inhibit the growth of undesirable plants or kill them.

Treatments with other osmolytes such as NaCl or mannitol allowed the osmolality to increase as much as with betaine treatment. However, the FT was not significantly improved when these osmolytes were used indicating that betaine specifically improves FT. These results suggest that the improvement in  $LT_{50}$  of 6°C cannot be explained solely by the osmolyte role of betaine (at a concentration of 250 mM, betaine would depress the freezing point of water by 0.32°C). We thus investigated whether betaine can induce a number of genes known to be associated with the development of FT. We examined the expression of three different proteins induced by low temperature using specific antibodies and immunoblot analysis as well as the expression of three other low temperature-induced genes using northern analysis. Our results showed that the protein WCOR410 (Genbank accession no. L29152) accumulates in a concentration-dependent manner when plants are exposed to varying amounts of betaine (Figure 3). When we examined the expression of the WCS120 (Genbank accession no. M93342) protein family or of the WCS19 (Genbank accession no. L13437) protein, we found that these proteins did not accumulate upon exposure to betaine (not shown). Since these two genes are known to be specifically induced by low temperature and that the gene *WCOR410* was inducible by low temperature, salinity, and water stress (19), we examined the possibility that other genes induced by drought or salinity are also induced by the presence of betaine. The genes *WCOR80* (Genbank accession no. U73212), *WCOR413* (Genbank accession no. U73216), and *WCOR825* (Genbank accession no. U73215) were only slightly inducible by betaine (not shown). These results suggest that the *WCOR410* gene is specifically induced to high levels by betaine exposure and may be a major contributor to the improvement of FT above the level that could be explained solely by the role of betaine as an osmolyte. The WCOR410 protein was found to be associated with the plasma membrane. This is the first abundant protein found in the membrane fraction of the vascular tissues (20), a region of the seedling known to be the most freezing sensitive tissue in wheat (21).

Betaine was shown to protect thylakoid membranes from freezing stress *in vitro* (22). Furthermore, in glycine-betaine deficient maize lines, high temperature decreases membrane stability and the resistance to photoinhibition as well as the steady-state yield of electron transport over PSII (23). These results suggest that betaine may confer greater membrane stability under both low and high temperature. It can also protect the photosystem II against salt stress *in vitro*. Since betaine is normally synthesized in the chloroplast, it may accumulate to a higher concentration

in this organelle as suggested (8, 14). Thus, we evaluated the effect of the exogenous supply of betaine on the resistance to photoinhibition and oxygen evolution.

During steady-state photosynthesis at the prevailing growth conditions, exposure of spring and winter wheat to 250 mM betaine resulted in small but consistently higher levels of qP and higher yields of PSII electron transport ( $\Phi_e$ ) than non-treated controls (Table I). Thus, betaine treated spring and winter wheat seedlings appeared to exhibit a greater capacity to prevent the reduction of PSII reaction centres than non-treated controls. The increased capacity to keep PSII reaction centres oxidized was correlated with a decreased susceptibility to low temperature (5°C) photoinhibition (measured according to 24) in betaine-treated seedlings. The Fv/Fm ratio of 250 mM betaine-treated plants submitted to high light (2h at 5°C and 1600  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) was of  $84 \pm 4\%$  of non-photoinhibited control plants compared to  $72 \pm 2\%$  for plants not treated with betaine. This effect of betaine on the photosynthetic machinery may improve the physiology and performance of the plants at low temperature and thus provide the plants with a greater capacity to use the available energy to develop FT.

Betaine application early in the fall improves the performance of golf turf and consequently increased winter survival. The betaine-treated turf showed a rapid regrowth in the spring indicating a higher winter survival rate and healthier plants at spring which have a better regrowth rate.

Based on the above results and based on the partial teachings of WO 95/35022, it is further expected that the germination rate of plant seeds can be improved at cold temperature. This method will comprise the steps of administering to the seeds an effective dosage regimen of betaine or derivative thereof, and allowing the same to germinate at cold temperatures. Such cold temperatures would be above about 0°C but not lower than the coldest temperature that the one plant seeds can withstand.

An important conclusion one can draw from these observations is that it may be possible to increase FT by using only one or two of the major genes (such as *Wcor410*) associated with this multigenic trait. Thus, it may be possible to increase the FT of cold sensitive species by transforming plants with this gene. Furthermore, our results suggest that FT could be improved in an additive manner in plants that already possess some degree of FT by overexpressing the most important genes involved in the process of FT. For example, overexpressing betaine dehydrogenase and choline monooxygenase under a low temperature promoter may allow the accumulation of betaine at the time where it could help against freezing stress. Such manipulations of betaine production could have a great agronomic and economic impact not only for protection against drought stress as previously suggested but also for FT as shown in this report. In addition, the results presented in this report indicate that exogenous

application of betaine before a predicted frost or sudden decrease in temperature may be exploited on a short-term basis to increase the resistance of cold sensitive plants to low temperature stress.

5 This invention has been described in details hereinabove, and it is readily apparent that modifications thereto can be made, without departing from the spirit of the invention and from the above teachings. These modifications fall under the scope of the invention as defined in the appended claims.

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**What is claimed is:**

1. A method of increasing or inducing cold or freezing tolerance in a plant, which comprises the following steps:

5 a) acclimating said plant to a temperature not lower than the coldest temperature that said plant is capable of withstanding, for a time sufficient to induce cold or freezing tolerance in said plant, and

b) increasing the concentration of betaine or a derivative thereof in said plant to induce cold or freezing tolerance in said plant;

10 whereby combined steps a) and b) increase or induce cold or freezing tolerance of said plant over and above the cold or freezing tolerance induced by each step alone.

2. A method as set forth in claim 1, wherein the increased or induced cold or freezing tolerance in said plant decreases the lethal temperature of said plant.

15 3. A method as set forth in claim 1, wherein the step of increasing the concentration of betaine or a derivative thereof includes administering a composition comprising betaine or a derivative thereof to said plant.

4. A method as set forth in claim 1, wherein the step of increasing the concentration of betaine or a derivative thereof includes overexpressing one or more genes involved in the synthesis of betaine or a derivative thereof.

20 5. A method as set forth in claim 4, wherein said one or more genes comprise betaine dehydrogenase or choline monooxygenase.

6. A method as set forth in claim 4, wherein said one or more genes are overexpressed under the control of a low temperature promoter.

7. A method as set forth in claim 1, wherein said temperature is higher than about 0°C.

25 8. A method as set forth in claim 1, wherein said temperature is below about 6°C.

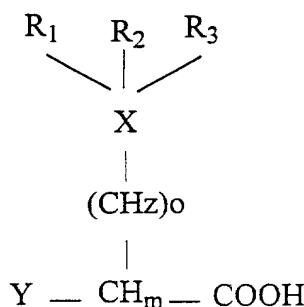
9. A method as set forth in claim 1, wherein steps a) and b) are substantially simultaneous.

10. A method as set forth in claim 1, wherein step b) precedes step a).

11. A method as set forth in claim 1, wherein step b) follows step a).

12. A method as set forth in claim 1, wherein said betaine or the derivative thereof comprises glycine betaine.

5 13. A method as set forth in claim 1, wherein said betaine or the derivative thereof comprises a compound of the formula



wherein X is N or S

z is 1 or 2

o is 0 or 1

m is 1 or 2

R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> are independently void or hydrogen or methyl

Y is void or Q-CH<sub>w</sub>-

wherein w is 1 or 2,

Q is a molecule of 2 to 9 carbon atoms comprising or not a ketone or a hydroxyl group, which may comprise 1 or 2 nitrogen or sulfur atoms and which may form a heterocycle alone or with X.

14. A method as set forth in claim 1, wherein said betaine or derivative thereof is selected from Glycinebetaine, β-alaninebetaine, 2-trimethylamino-6-ketoheptanoate, prolinebetaine, proline, N-methyl-L-proline, *trans*-4-hydroxy-N-methyl-L-proline, *cis*-3-hydroxy-N-methyl-L-proline, (-)4-hydroxyproline betaine, (+)4-hydroxyprolinebetaine, 3-hydroxyprolinebetaine, histidinebetaine, tryptophanbetaine, 2-mercaptohistidinebetaine, pipecolabetaine and nicotinic acid betaine.

15. A method as set forth in claim 1, wherein said plant is selected from the group consisting of tropical plants, gramineae and grasses.

16. A method as set forth in claim 15, wherein said plant is barley or wheat.
17. A method as set forth in claim 15, wherein said plant is golf turf.
18. A method as set forth in claim 10 or 11, wherein said composition comprises glycine betaine at a concentration lower than about 500 mM.
- 5 19. A method as set forth in claim 12, wherein said concentration is about 250 mM or lower.
20. A method as set forth in claim 13, wherein said concentration is about 250 mM.
21. A method as set forth in claim 17, wherein the method increases winter survival, spring regrowth, greening or density of golf turf.
- 10 22. A method as set forth in claim 20, wherein the increase in freezing tolerance is by at least about 5°C.
23. A method as set forth in claim 1, wherein the cold or freezing tolerance induced by said each step alone or in combination is due at least in part to an increased accumulation of a protein having the biological characteristics of WCOR410 protein.
- 15 24. A method as set forth in claim 1, which further results in improving photosynthetic capacity and overall physiology of said plant at cold temperature.
25. A method as set forth in claim 1, which further results in increasing or inducing tolerance of salinity, water stress, or both, in said plant.
- 20 26. A method of reducing the growth rate of a plant, thus having a growth-retarding effect, which comprises the step of treating said plant with an effective amount of a composition comprising betaine or derivative thereof, which composition is not lethal to said plant.
27. A method as set forth in claim 26, wherein said dosage regimen of betaine or derivative thereof is not toxic to said plant.
- 25 28. A method as set forth in claim 26 or 27, wherein said plant is selected from gramineae and grasses.

29. A method as set forth in claim 28, wherein said plant is golf turf.

30. A method as set forth in claim 28, wherein said plant is wheat or barley.

31. A method as set forth in claim 28, wherein said dosage regimen in growing said plant in the presence of betaine is at a concentration of 500 mM or lower for four days,  
5 which results in a growth rate reduction by about 75%.

32. A method of stimulating and improving the germination rate of plant seeds at a temperature not lower than the coldest temperature that said plant seeds can withstand, which comprises the steps of administering to said seeds an effective amount of a composition comprising betaine or a derivative thereof, and allowing said seeds to  
10 germinate at said temperature.

33. A method of inducing or increasing cold tolerance in a plant sensitive to temperature of about 0°C or higher, which comprises the step of administering a composition comprising betaine or a derivative thereof to said plant prior to a decrease in temperature.

34. A method of increasing or inducing cold or freezing tolerance in a plant, which  
15 comprises the step of increasing the accumulation of a protein having the biological characteristics of WCOR410 in said plant.

35. A method as set forth in claim 34, wherein said protein having the biological characteristics of WCOR410 is increased by increasing the concentration of betaine or a derivative thereof in said plant.

20 36. A method as set forth in any one of claims 1, 33 and 34, wherein each mention of said cold or freezing tolerance comprises about an optimal cold or freezing tolerance.

37. A method as set forth in any one of claims 23, 34 or 35, wherein the protein is WCOR410.

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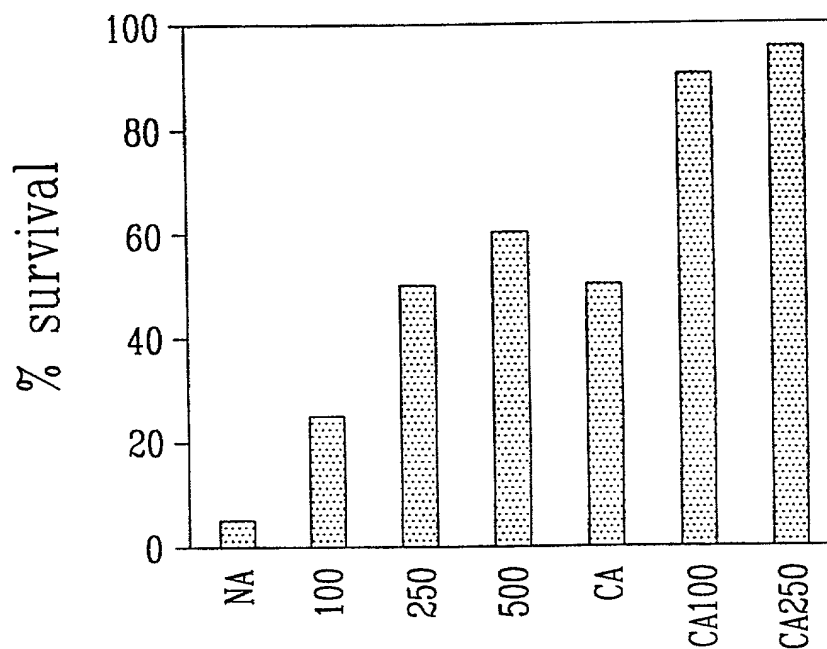


FIGURE 1A

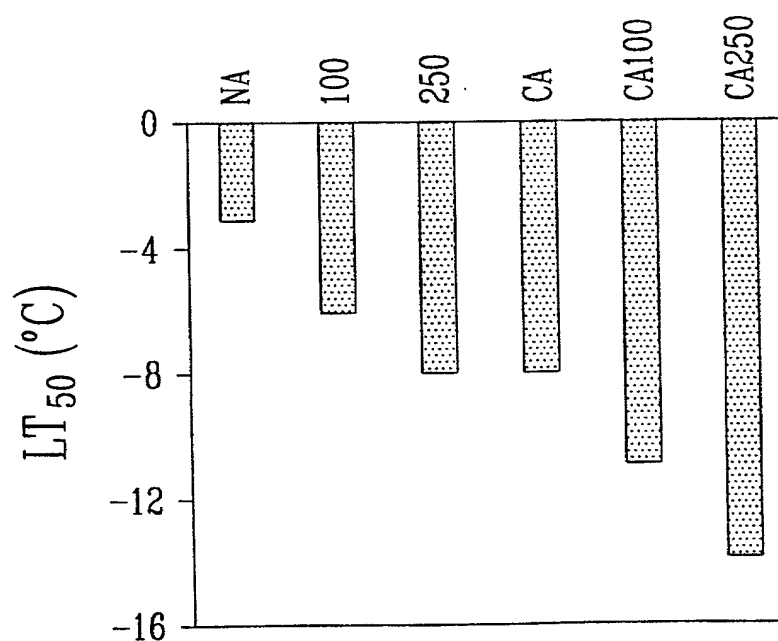


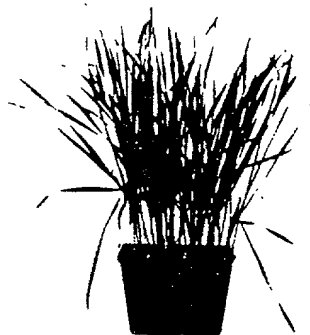
FIGURE 1B

**A**

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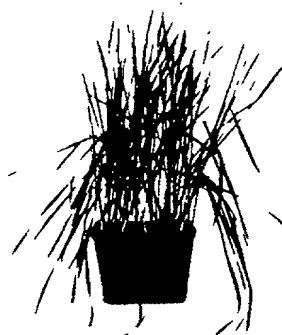


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**SUBSTITUTE SHEET (RULE 26)**



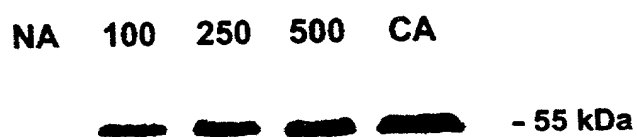


FIGURE 3

09/446722

Figure 4 A

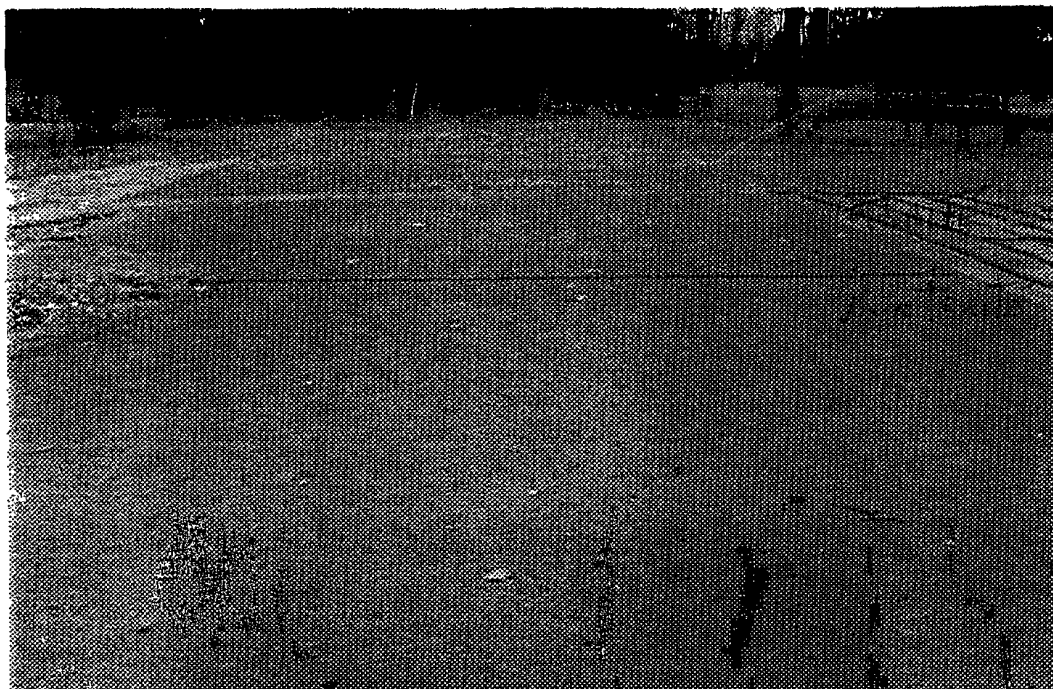
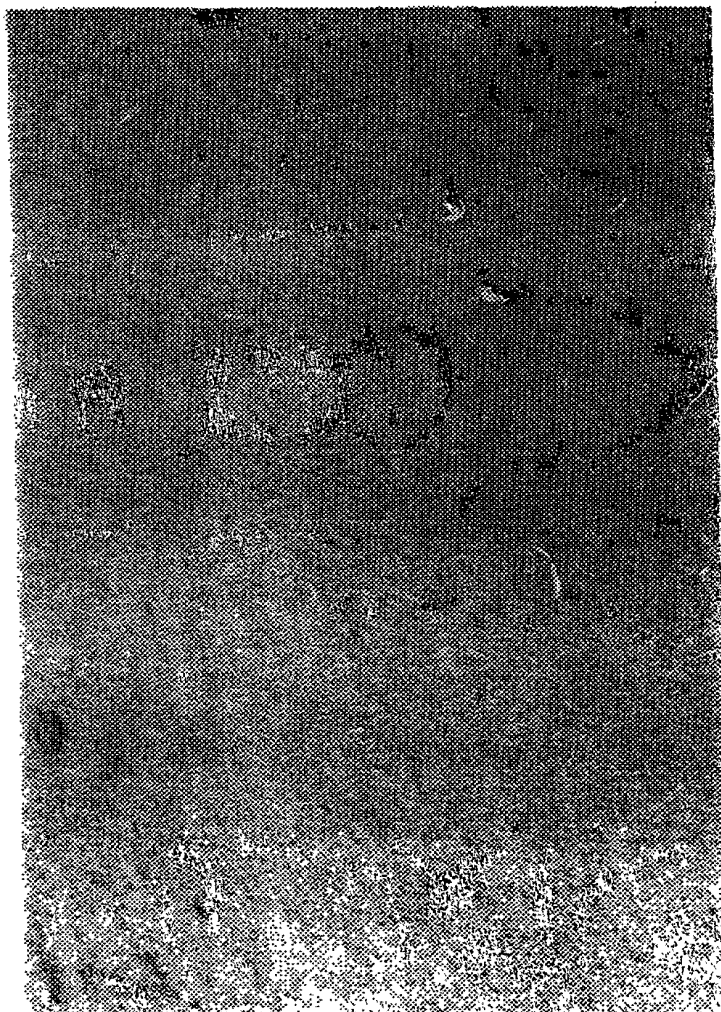
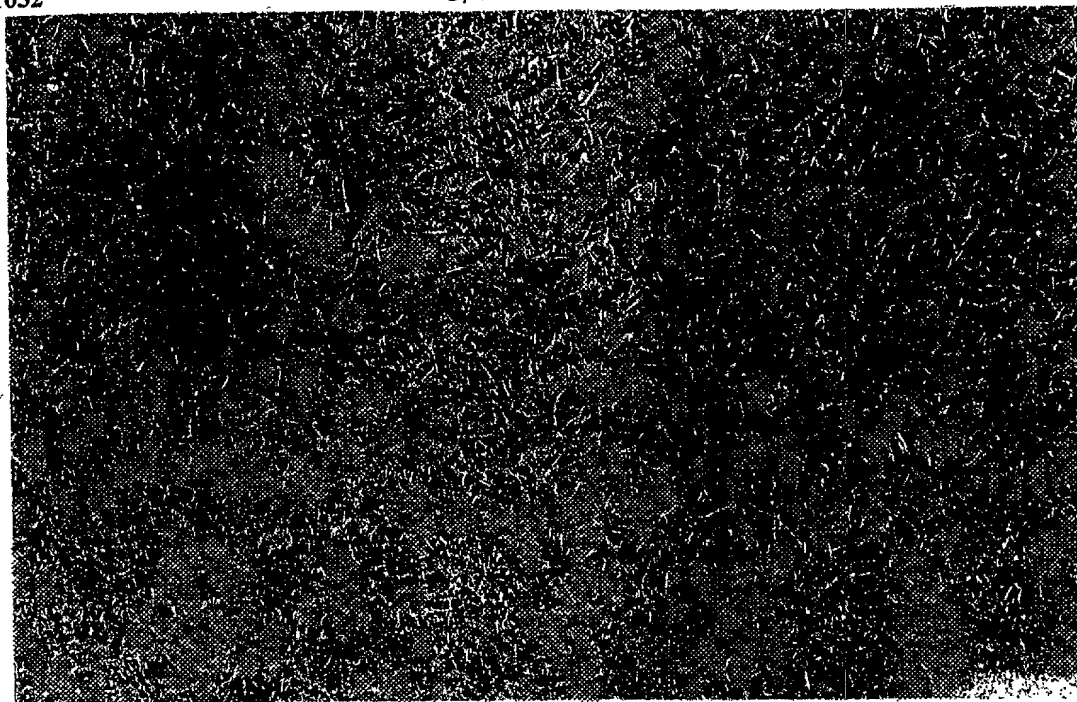


Figure 4 B



00E040" T1/294450

Variable	Mean	SD	Min	Max
Age	35.5	10.5	18	65
Gender	Male	100%		
Marital Status	Married	100%		
Education	High School	100%		
Occupation	Farmer	100%		
Religion	Islam	100%		
Income	Low	100%		
Health Status	Good	100%		
Smoking Status	Non-smoker	100%		
Alcohol Consumption	None	100%		
Stress Level	Low	100%		
Sleep Quality	Good	100%		
Dietary Habits	Healthy	100%		
Exercise Frequency	Regular	100%		
Family Size	Small	100%		
Community Support	High	100%		
Access to Healthcare	Good	100%		
Health Insurance	Yes	100%		
Genetic History	None	100%		
Chronic Conditions	None	100%		
Medication Use	None	100%		
Psychological Health	Stable	100%		
Social Interaction	Active	100%		
Work-Life Balance	Good	100%		
Overall Well-being	High	100%		



**Figure 5 B**



**Combined Declaration for Patent Application and Power of Attorney**

(Includes Reference to PCT International Applications)

Attorney's Docket Number

P04334US0

As a below named inventor, I hereby declare that:  
My residence, post office address and citizenship are as stated below next to my name.  
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS**

the specification of which (check only one item below)

- ☐ is attached hereto.  
☐ was filed as United States application

Serial No. \_\_\_\_\_

on \_\_\_\_\_

and was amended

on \_\_\_\_\_ (if applicable).

☒ was filed as PCT international applicationNumber **PCT/CA98/00648**on **2 JULY 1998 (02.07.98)**

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:**

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
CA	2,209,591	3 JULY 1997 (03.07.97)	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:**

U.S. APPLICATIONS			STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			



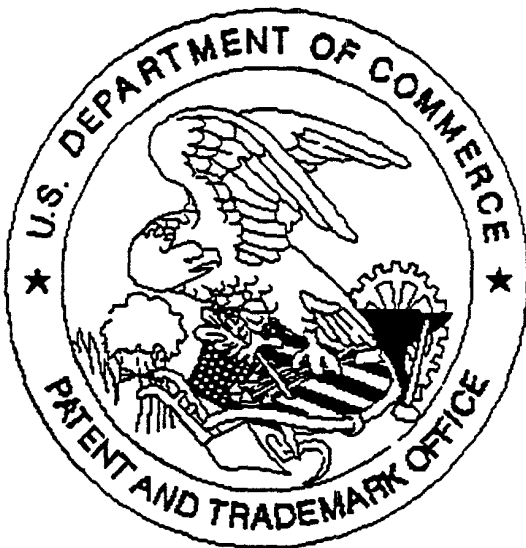
Combined Declaration for Patent Application and Power of Attorney (Continued) (Includes Reference to PCT International Applications)		Attorney's Docket Number <b>P04334US0</b>	
<b>POWER OF ATTORNEY:</b> As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. ( <i>List name and registration number</i> ) Donald H. Zarley, Reg. #18,543; Bruce W. McKee, Reg. #19,651; Dennis L. Thomte, Reg. #22,497; Michael G. Voorhees, Reg. #25,715; Edmund J. Sease, Reg. #24,741; Mark D. Hansing, Reg. #30,643; Kirk M. Hartung, Reg. #31,021; Daniel J. Cosgrove, Reg. #36,770; Michael R. Crabb, Reg. #37,298; Heidi Sease Nebel, Reg. #37,719; Wendy K. Marsh, Reg. #39,705; Jeffrey D. Harty, Registration No. 40,639; James A. Napier, Registration No. 42,025; Mark Ziegelbein, Registration No. 43,307; Timothy J. Zarley, Registration No. P-45,253; and Patty L. Ades, Registration No. P-44,496.			
Send correspondence to: Heidi S. Nebel <u>ZARLEY, MCKEE, THOMTE, VOORHEES &amp; SEASE</u> <u>801 Grand Avenue, Suite 3200</u> <u>Des Moines, Iowa 50309-2721</u>		Direct Telephone Calls to: (Name and telephone number) Heidi S. Nebel 515-288-3667	

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3	POST OFFICE ADDRESS	POST OFFICE ADDRESS <b>430 RICHARD</b>	CITY <b>LONGUEUIL</b>	STATE & ZIP CODE/COUNTRY <b>QUEBEC J4L 2A4 CA</b>
2	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
0	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
4	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
2	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
0	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
5	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
2	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
0	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
6	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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